1997 Gene Therapy Meetings

Domestic

September 4–6, 1997 Chapel Hill, NC	International Symposium on Gene Therapy for Hemophilia University of North Carolina at Chapel Hill (919) 962-2118, Fax (919) 966-1664 gtmeet@med.unc.edu http://www.med.unc.edu/thromb/gene
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November 22, 1997 San Diego, CA	Sixth Annual Conference on Gene Therapy of Cancer Sidney Kimmel Cancer Center (619) 565-9921 http://www.pcmisandiego.com/pcminc/gene.htm

International

September 11–13, 1997 Berlin, Germany	3rd European Conference on Gene Therapy of Cancer Humboldt-University, Charite, Berlin, Germany +49-30-38370-751, Fax +49-30-38370-789 post@blackwell-gem.de http://www.blackwell-gem.de
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LacZ and Interleukin-3 Expression *In Vivo* after Retroviral Transduction of Marrow-Derived Human Osteogenic Mesenchymal Progenitors

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ABSTRACT

Human marrow-derived mesenchymal progenitor cells (hMPCs), which have the capacity for osteogenic and marrow stromal differentiation, were transduced with the myeloproliferative sarcoma virus (MPSV)-based retrovirus, vM5LacZ, that contains the LacZ and neo genes. Stable transduction and gene expression occurred in 18% of cells. After culture expansion and selection in G418, approximately 70% of neo^r hMPCs co-expressed LacZ. G418-selected hMPC retain their osteogenic potential and form bone *in vivo* when seeded into porous calcium phosphate ceramic cubes implanted subcutaneously into SCID mice. LacZ expression was evident within osteoblasts and osteocytes in bone developing within the ceramics 6 and 9 weeks after implantation. Likewise, hMPCs transduced with human interleukin-3 (hIL-3) cDNA, adhered to ceramic cubes and implanted into SCID mice, formed bone and secreted detectable levels of hIL-3 into the systemic circulation for at least 12 weeks. These data indicate that genetically transduced, culture-expanded bone marrow-derived hMPCs retain a precursor phenotype and maintain similar levels of transgene expression during osteogenic lineage commitment and differentiation *in vivo*. Because MPCs have been shown to differentiate into bone, cartilage, and tendon, these cells may be a useful target for gene therapy.

OVERVIEW SUMMARY

We have characterized human bone marrow-derived osteogenic mesenchymal progenitor cells (hMPCs) as potential targets for gene transfer. hMPCs were readily transduced with a myeloproliferative sarcoma virus-based retroviral vector. After *in vitro* selection and expansion of neo^r-expressing cells in G418, the expression of a second unselected gene, LacZ or interleukin-3 (IL-3), was maintained. Transduced, G418-selected hMPCs retained their osteogenic precursor phenotype *in vivo* in a SCID mouse xenograft model of osteogenesis, and continued high expression of the LacZ gene in osteoblasts and fully differentiated osteocytes. Likewise, hIL-3-transduced hMPCs, placed within the same osteoconductive microenvironment, secreted hIL-3 into the systemic circulation. Thus, hMPCs are a unique cellular vehicle for *ex vivo* gene therapy directed toward mesenchymal tissues.

INTRODUCTION

Bone marrow (bm) is a complex microenvironment that contains at least two stem and progenitor cell populations. The first population consists of hematopoietic stem cells (HSC) and their multipotential progeny, which differentiate into all circulating blood cells of the lymphoid, myeloid, and erythroid lineages. The second precursor pool consists of mesenchymal progenitor cells (MPCs), which have the capacity to differentiate into a variety of mesenchymal phenotypes, including osteoblasts (Goshima *et al.*, 1991a, b; Nakahara *et al.*, 1991, 1992; Haynesworth *et al.*, 1992; Prockop, 1997), chondrocytes (Naka-

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bara *et al.*, 1991, 1992) tendon (Caplan *et al.*, 1993), muscle (Wakitani *et al.*, 1995), and bone marrow stromal fibroblasts (Friedenstein, 1980; Owen, 1985). The MPC is derived from the marrow stromal fibroblast population, but has been uniquely identified by its reactivity with a series of monoclonal antibodies, SH2, SH3, and SH4 (Haynesworth and Caplan, 1992), and by the capacity to differentiate into specific lineages as noted above. Although gene transduction of primary human HSCs has been described, few studies have addressed the use of primary human marrow-derived mesenchymal cells and no studies have demonstrated maintenance of a precursor phenotype following gene transfer nor the ability of the transduced gene to be expressed following differentiation *in vivo*. Nolta *et al.* (1994) transduced human stromal cells with interleukin-3 (IL-3), infused them into immunodeficient mice, and noted persistent IL-3 in the circulation. However, the osteogenic capacity of these cells and their fate *in vivo* were not determined. More recently, this group demonstrated the ability of IL-7-transduced stromal cells to enhance immune reconstitution (Bolotin, 1996).

MPCs are derived from low-density adherent BM fibroblastic cells that can be culture expanded in culture from many species, including rat (Goshima *et al.*, 1991a,b; McCulloch *et al.*, 1991; Dennis *et al.*, 1992), mice (Friedenstein, 1976), rabbit (Owen *et al.*, 1987), dog, guinea pig (Friedenstein *et al.*, 1970), and human (Nakahara *et al.*, Bab *et al.*, 1988; Haynesworth *et al.*, 1992; Bruder *et al.*, 1996). MPCs isolated from a BM adherent cell population retain their undifferentiated precursor phenotype during expansion, and differentiate along one or more mesenchymal lineages in response to the appropriate stimuli (Owen *et al.*, 1987; Bab *et al.*, 1988; Caplan *et al.*, 1993). Culture-expanded MPC populations seeded into porous calcium phosphate ceramic cubes and implanted subcutaneously in immunodeficient mice differentiate along the osteogenic lineage to form functional osteoblasts and osteocytes and, when densely packed, chondrocytes (Goshima *et al.*, 1991a,b; Nakahara *et al.*, 1991, 1992; Dennis *et al.*, 1992; Haynesworth *et al.*, 1992). Bone formed within the ceramic is derived from the donor MPCs (Friedenstein *et al.*, 1970; Haynesworth *et al.*, 1992a), and shows dynamics reminiscent of normal bone development, including remodeling and the formation of a hematopoietic microenvironment *in vivo* (Goshima *et al.*, 1991a,b,c; Haynesworth *et al.*, 1992a). In addition, cytokines expressed by human MPCs (hMPCs) reveal characteristics of both stromal and osteogenic cells. Macrophage colony-stimulating factor (M-CSF), stem cell factor (SCF), IL-6, IL-11, and leukemia inhibitory factor (LIF) are constitutively produced in the supernatant of hMPC cultures and granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) can be induced by IL-1α (Haynesworth *et al.*, 1996). MPCs are also capable of supporting hematopoietic progenitors in long-term culture, an indication of their stromogenic potential (Majumdar *et al.*, 1995). Thus, MPCs have plasticity to be both osteogenic, chondrogenic, and supportive of hematopoietic cells as stromal elements of the marrow microenvironment.

We report that hMPCs transduced with a retroviral vector and culture expanded for up to 6 weeks *in vitro* express the transduced gene products *in vitro* and *in vivo*, and retain their ability to form bone *in vivo* when placed in an osteogenic ceramic cube microenvironment.

MATERIALS AND METHODS

Bone marrow harvest

BM aspirates (10 ml from two 5-ml aspirations) were obtained from the posterior iliac crest of adults who had given informed consent under an IRB-approved protocol to the Hematopoietic Stem Cell Facility of the Case Western Reserve University Ireland Cancer Center. Although a small amount of peripheral blood typically is aspirated along with the marrow-derived cells, we have recently shown (Lazarus *et al.*, 1997) that the peripheral blood does not contain MPCs. All marrow samples were histologically normal.

Preparation and propagation of marrow-derived human MPCs

Preparation of the adherent marrow-derived cells has previously been described (Haynesworth *et al.*, 1992a). Briefly, single-cell suspensions of bone marrow were layered on 70% (Sigma, St. Louis, MO) gradients and low-density mononuclear cells were recovered. Fifty × 10^6 cells were plated in Dulbecco's modified Eagle's medium (DMEM) + 10% fetal bovine serum (FBS), prescreened for growth and maintenance of the osteogenic potential of hMPCs, as described (Goshima *et al.*, 1991b,c; Lennon *et al.*, 1995), on 100-mm^2 plastic tissue culture dishes at 37°C, 5% CO_2. After 3 days, the medium was changed to remove nonadherent hematopoietic cells. Thereafter, the medium was changed twice weekly. Approximately 10–12 days after primary culture, the cells were detached from the plate with 0.25% trypsin containing 1 m*M* EDTA (GIBCO) for 5 min at 37°C. They were diluted 1:3 and cyclically replated in fresh medium when cells reached 80% confluence.

vM5LacZ and viral collection

vM5LacZ (also termed vM5lac; Allay *et al.*, 1995) contains the bacterial β-galactosidase (β-Gal) gene (LacZ) and the neomycin phosphotransferase gene (neo) both under the transcriptional control of the MPSV 5′ long terminal repeat (LTR) (kindly provided by W. Ostertag) as previously described by Clapp *et al.* 1995). Amphotropic vM5LacZ producers were obtained by infecting GP + envAm12 retroviral packaging cells (Markowitz *et al.*, 1988) with supernatant from LacZ-expressing GP + E86 ecotropic cells (Clapp *et al.*, 1995) using "ping-pong" proviral amplification (Bodine *et al.*, 1990), followed by G418 selection. A clone transmitting a high neo^r titer (1 × 10^6 neo^r CFU/ml) and β-Gal activity to NIH-3T3 was chosen and used in all subsequent gene transfer experiments.

vL-IL-3SN

vL-IL-3-SN (kindly provided by Drs. D. Kohn and J. Nolta, USC) has previously been described (Nolta *et al.*, 1994). Supernatant from vL-IL-3SN producer cells was used to infect GP + envAM12 cells, which were then clonally expanded in G418. Supernatant from a clone transmitting a titer of 3 × 10^6 neo^r CFU/ml was collected as described above and utilized for subsequent gene transfer experiments. Amphotropic virus was collected every 18–24 hr for 6 consecutive days from producer cells when 80% confluent (Allay *et al.*, 1995).

Helper virus assays

Supernatants from both vM5LacZ- and vIL3-SN-transduced NIH-3T3 and hMPCs and serum from mice implanted with transduced hMPC were used to infect NIH-3T3 cells to detect infectious virus or NIH-LacZ cells (a NIH-3T3 cell line containing a vM5LacZ provirus) to detect virus capable of proviral rescue as previously described (Allay *et al.*, 1995). At no time was replication-competent retrovirus derived from vM5LacZ or v-IL-3-SN detected by our assays with a limit of detection of approximately 2 × 10^6 CFU/ml.

Retroviral transduction of hMPC

hMPCs were grown in DMEM + 30% heat-inactivated (HI) FBS for 18–24 hr following first or second passage to increase cell proliferation and enhance the rate of gene transfer. Preliminary experiments indicated a higher degree of gene transfer when the cells were serum stimulated in 30% FBS than with 20% FBS, but formal comparison, with statistical analysis, was not performed. Because this is the first publication describing gene transfer into MPCs, there is no available published evidence to compare directly the two FBS preparations. Medium was replaced with 4 ml of 0.45-μm-filtered vM5LacZ or vL-IL-3-SN viral supernatant, containing 6 μg/ml Polybrene (Sigma, St. Louis, Missouri). After 6 hr, viral supernatant was removed and cells were cultured in DMEM + 30% HI FBS (which resulted in a higher level of gene transfer than 20% FBS) for 18 hr and repeated daily for 4 days. Cultures of transduced hMPC were either X-Gal-stained (see below) to determine the frequency of vM5LacZ infection and gene expression, or trypsinized and replated at clonal density in G418 to determine the number of clonal cells expressing the proviral genes, or expanded in G418 for further experiments. For all *in vivo* experiments, transduced cell populations, not individual clones, were used.

SH2 monoclonal antibody staining of MPCs

Cultured MPCs were stained with the MPC-specific monoclonal antibody, SH2, as we have previously described (Haynesworth and Caplan, 1992).

X-Gal staining of hMPC

vM5LacZ-transduced or untransduced hMPC were fixed in freshly prepared 2% formaldehyde, 0.2% glutaraldehyde in phosphate-buffered saline (PBS) for 5 min at 4°C, washed and stained in fresh 1 mg/ml X-Gal in 20 m*M* potassium ferrocyanide, 20 m*M* potassium ferricyanide, and 2 m*M* $MgCl_2$ in PBS (Sanes *et al.*, 1986) and counterstained with 0.1% crystal violet (Lennon *et al.*, 1995). To assay the differentiation status of vM5LacZ-transduced hMPCs, we examined the reactivity of these cells with the SH2 antibody (Haynesworth *et al.*, 1992), which was raised against culture-expanded hMPCs.

Preparation of ceramics and surgical implantation

Four- to 6-week culture expanded, 5 × 10^4 retrovirally transduced hMPCs selected in 0.5 mg/ml G418 (vM5LacZ- or vL-IL-3-SN-transduced hMPCs), or untransduced hMPCs (control hMPCs) were seeded into 3-mm porous tricalcium phosphate/hydroxyapatite (40:60) ceramics coated with human fibronectin (McCulloch *et al.*, 1991) and implanted subcutaneously into CB.17/SCID (Harland) immunodeficient mice as previously described (Dennis *et al.*, 1992). NOD/LtSz-scid/scid mice (Jackson Labs) were used for implantation with ceramics seeded with vL-IL-3-SN-transduced hMPCs and were infused 1 week later with 1 × 10^7 human cord blood derived mononuclear cells to assess the effects of hIL-3 production on human hematopoiesis. These mice were analyzed 2–12 weeks after implantation. The NOD/LtSz-scid/scid strain was used because it has been shown to be more permissive to reconstitution with human hematopoietic cells than the SCID strain (Greiner *et al.*, 1995).

β-Gal detection of LacZ$^+$ cells in ceramics

Six and nine weeks after implantation, ceramics were recovered, trimmed of excess mouse tissue, fixed in freshly prepared 2% formaldehyde, 0.2% glutaraldehyde in PBS at 4°C for 1 hr, rinsed, and stained with X-Gal as above. Ceramics were then demineralized in Rapid Bone Decalcifier (Dupage Kinetics Laboratories, Inc Plainfield, IL) and embedded in paraffin. LacZ$^+$ ceramics turn bright blue, whereas, cubes coated with control cells did not stain blue. Six-micrometer serial sections were counterstained with Neutral Red, Mallory Heidenhain (for bone identification) (Humanson, 1972), or Hematoxylin and Eosin. Bone is identified as deep blue staining matrix with Mallory Heidenhain staining (Dennis *et al.*, 1992).

Northern analysis

Total RNA was isolated from vM5LacZ, G418-selected hMPC snap-frozen in liquid nitrogen by lysing cells in guanidinium thiocyanate and layering onto cesium chloride cushions (Sambrook *et al.*, 1989). RNA (10 μg) was electrophoresed on a denaturing formaldehyde gel, blotted onto Gene Screen Plus (DuPont), and hybridized with a 400-bp oligonucleotide neo probe that recognizes both the full-length 7.5-kb and the spliced 2.5-kb proviral transcript.

hIL-3 quantitation

hIL-3 levels in culture supernatant and plasma severe combined immunodeficiency (SCID) mice were measured by an enzyme-linked immunosorbent assay (ELISA) kit in triplicate with normal mouse serum as control (R&D Systems, Minneapolis, MN). hIL-3 expression was normalized for cell number as previously described (Haynesworth *et al.*, 1996).

RESULTS

Production of amphotropic vM5LacZ retrovirus

Amphotropic virus containing supernatant was collected daily for 6 days from producer cells once they were 80% confluent. The neo-resistant viral titer increased 10-fold over a 6-day period from 1.3 ± 0.4 × 10^5 CFU/ml (n = 3) on day 1 to 1.3 ± 0.9 × 10^6 CFU/ml (n = 6) on day 6. The co-expression of the LacZ gene detectable by X-Gal staining in transduced

NIH-3T3 cells selected in G418 ranged from 31% to 78% ($n = 8$).

vM5LacZ transduction of hMPCS in vitro

First- or second-passage hMPCs were infected with vM5LacZ and analyzed for transduction and expression of the lacZ and neo transgenes. A Northern blot of vM5LacZ transduced, G418 selected hMPCs (Fig. 1) identified both full-length 7.5-kb and spliced 2.5-kb viral transcripts at a ratio of 1.9:1. Thus, vM5LacZ-transduced hMPC cultures contain cells that transcribe both LTR-derived transcripts. As can be seen in Fig. 2A, vM5LacZ-transduced, G418-selected hMPCs remained a morphologically homogeneous population of fibroblastic cells, with about 70% of cells within the colony co-expressing LacZ as indicated by blue staining after X-Gal exposure. The cells stained uniformly with SH2, a monoclonal antibody that selectively recognizes cultured hMPCs (Haynesworth and Caplan, 1992; Fig. 2B). Transduction efficiency was estimated by G418 resistance and X-Gal staining. In 9 separate experiments, a mean of 18 ± 4% of the cells stained blue compared to none of the nontransduced cells. To assess gene transfer indepen-

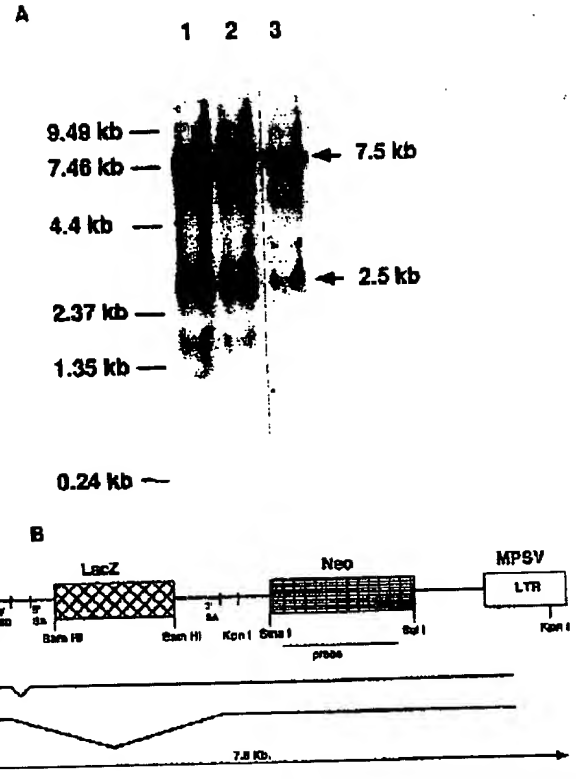

FIG. 1. vM5LacZ expression in transduced hMPC *in vitro*. A. vM5lacZ-transduced hMPC grown in 0.5 mg/ml G418 were analyzed by Northern blot, which identified 1.9-fold greater expression of the full-length 7.5-kb transcript relative to the spliced 2.5-kb transcript by relative density. Lane 1, vM5LacZ-transduced hMPC from donor USN 461; lane 2, vM5LacZ transduced hMPC from donor USN 475; lane 3, vM5LacZ-transduced NIH-3T3 cells. B. Map of the vM5LacZ provirus, indicating the full-length 7.5-kb and spliced 2.5-kb transcripts. The PCR-generated fragment within the neo coding region used as Northern probe is shown.

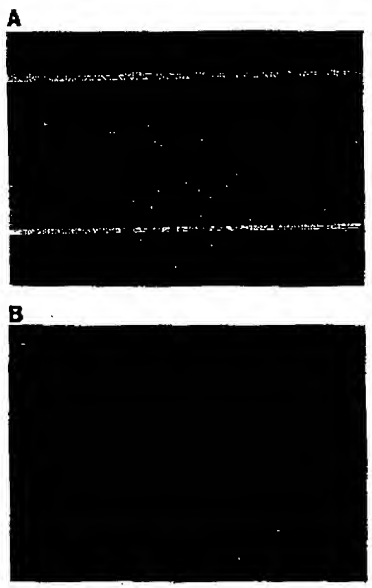

FIG. 2. Morphologic appearance of MPCs. A. Cultured MPCs transduced with vM5LacZ and selected in G418 were stained with X-Gal revealing LacZ expression in a high proportion of cells and a morphologically homogeneous population of fibroblastic cells. B. Cells were stained with SH2 mouse antihuman MPC monoclonal antibody followed by FITC goat anti-mouse antibody and examined under fluorescence microscopy. Original magnification, 100×. SH2-negative cell preparations of NIH-3T3 cells or human blood mononuclear cells do not stain at all and would appear black under these conditions (Haynesworth *et al.*, 1992).

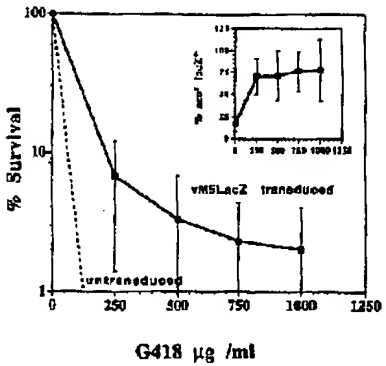

FIG. 3. G418 resistance and LacZ co-expression in vM5LacZ-transduced hMPCs *in vitro*. hMPCs were infected with vM5LacZ, plated in increasing concentrations of G418, and scored for clonal survival relative to an untreated control culture of hMPCs. The insert indicates the proportion of X-Gal staining of colonies based on the same G418 dose range of the x axis. The curve shows the degree of LacZ and neo co-expression.

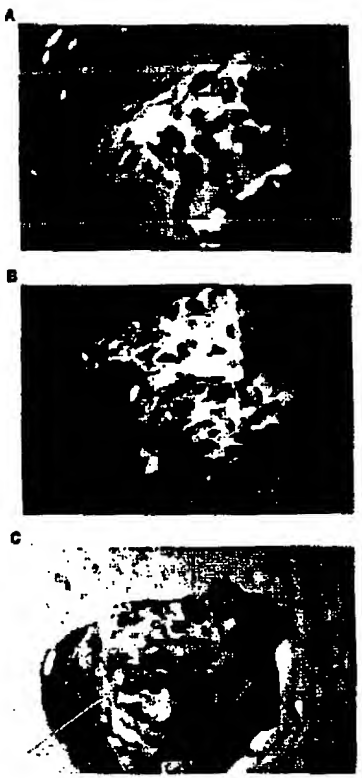

FIG. 4. Ceramic cubes after subcutaneous implantation. Porous calcium phosphate ceramic cubes were seeded with vM5LacZ-transduced or control hMPCs, implanted subcutaneously in SCID mice, and sacrificed 6 weeks later. A. *In situ* photomicrograph showing the extensive vascular network surrounding a ceramic loaded with vM5LacZ-transduced hMPCs. B and C. X-Gal stain of entire ceramic. Blue color indicates LacZ expression. B. Ceramic loaded with vM5LacZ-transduced MPCs stains blue. C. Ceramic loaded with untransduced hMPCs does not stain with X-Gal.

density of lacZ, we used G418 resistance, which measures functional expression of neo and represents a lower limit of transfection based on expression of neo at a sufficient level to result in G418 resistance, not retroviral gene transfer. Figure 3 shows neo+ survival of vM5LacZ-transduced hMPCs plated at clonal density, indicating that approximately 7 ± 5% ($n = 9$ experiments) of the colonies survived 0.25 mg/ml G418 compared to none of the untransduced cells. The actual gene transfer rate was not determined at this point because it was difficult to isolate distinct colonies of cells for polymerase chain reaction (PCR) analysis of provirus, as is conventional, without some cross-contamination of the colonies with cells from adjacent colonies. Thus, the transduction efficiency is 18% by X-Gal staining for lacZ expression and 7% by G418 resistance as a measure of neo expression. The proportion of LacZ-expressing colonies was 70 ± 28% in cultures selected for G418 resistance (Fig. 3). These data imply indirectly that at least 5% of cells prior to selection expressed both lacZ and neo and that up to 13% of cells expressed detectable levels of lacZ but not neo. After neo selection, all cells were G418 resistant and, in this setting, most also expressed lacZ.

Maintenance of progenitor potential and gene expression in vM5LacZ-transduced hMPCs in vivo

vM5LacZ-transduced and untransduced hMPCs from the same donors were assayed in SCID mice for their potential to differentiate into bone forming cells as described (Haynesworth *et al.*, 1992). This model is different than the previously described infusion of marrow fibroblasts (Nolta *et al.*, 1994) in that an ectopic site was used to promote differentiation along the osteogenic lineage (Haynesworth *et al.*, 1992). hMPCs seeded into calcium-phosphate ceramics and implanted subcutaneously in CB17/SCID mice were analyzed. A total of 41 separate ceramic cubes were seeded with hMPCs from 7 different human donors. Thirty-two of these ceramics were seeded with vM5LacZ-transduced hMPCs (from all 7 donors), whereas 9 of these ceramics were seeded with contransduced (control) hMPCs (from 3 of the 7 donors).

Six and 9 weeks after implantation, ceramics were recovered and examined histologically for bone and presence of LacZ+ cells. At both times points, macroscopic examination of the ceramics revealed a vascular network surrounding the implanted ceramic (Fig. 4A). After dissection from the host connective tissue, all ceramics were X-Gal stained. The pores of ceramics seeded with vM5LacZ-transduced hMPCs were a distinct blue, indicating the presence of LacZ+ cells, whereas ceramics seeded with control hMPCs showed no color change (Fig. 4B, C).

Each ceramic was examined histologically, with a minimum of 24 sections per cube. None of the 8 ceramics seeded with NIH-3T3 cells and none of the ceramics not seeded with cells and implanted in the SCID for either 6 or 9 weeks exhibited bone formation within the ceramics (data not shown), confirming previous studies indicating the requirement for adherent MPCs for bone formation to take place in this model (Goshima *et al.*, 1991a,b; Dennis *et al.*, 1992).

Bone formation and LacZ expression were evaluated in the ceramics after X-Gal staining, sectioning, and histological processing by counterstaining with Neutral Red, Mallory Heidenhain, or Hematoxylin and Eosin, none of which precluded identification of X-Gal-stained cells (the latter two counterstains turn X-gal-staining cells a darker bluish-purple). In the 6-week group, bone was detected by Mallory Heidenhain staining in 12 of 20 ceramics seeded with vM5LacZ-transduced hMPCs (derived from 3 of 7 donors) and 6 of 7 ceramics seeded with control hMPCs (derived from 2 of 3 donors). Analysis of paired hMPC ceramics from cultures of cells from 3 donors revealed that bone formation was observed concurrently in implants from the same 2 of 3 donors, indicating that the osteogenic potential of hMPCs was not affected by vM5LacZ transduction. This degree of heterogeneity in bone formation has been previously noted (Goshima *et al.*, 1991 a,b; Dennis and Caplan, 1996; Dennis *et al.*, 1992).

In the 9-week group, bone was detected in 4 of 12 ceramics seeded with vM5LacZ-transduced hMPCs and 1 of 4 ceramics seeded with untransduced MPCs. Among the ceramics studied, there were five paired sets from the same donors of vM5LacZ transduced hMPCs harvested at both 6 and 9 weeks. In the two sets, bone formation was detected at both 6 and 9 weeks. In two other sets, bone formation was not detected at either time, and, in the last set, bone formation was detected only at 9 weeks.

Figure 5 shows photomicrographs of bone-containing ceramics seeded with vM5LacZ-transduced hMPCs (Fig. 5A–D) or control hMPCs (Fig. 5E,F). Osteoblasts were either cuboidal or fusiform cells at the edge of bone, whereas osteocytes were embedded within bony lacunae. Figure 5, A–D, shows ceramics seeded with vM5LacZ-transduced hMPCs which contain X-Gal-stained blue LacZ+ osteoblasts and osteocytes encased within bone. Ceramics seeded with control hMPCs had no detectable X-Gal staining in sections counterstained by Neutral Red. Hematoxylin & Eosin-stained sections are shown for ease of viewer observation (Fig. 5E,F). Many vM5LacZ-transduced hMPCs differentiated into osteogenic cells (Fig. 5A–D) and expressed lacZ by X-Gal stain. This suggests that hMPCs give rise to osteoblasts and osteocytes, as well as cells that may retain a less mature phenotype or have yet to commit to a lineage. Most of the cells in the middle of the ceramic pore, not up against the ceramic, are host-derived connective tissue cells. However, an occasional X-Gal + cell was noted within these spaces in cubes containing vM5LacZ-transduced hMPCs. Thus, there appears to be some ability of the ceramic-attached cells to migrate into the ceramic space, although there was no evidence that a mature stromal space was generated.

FIG. 5. LacZ expression and bone formation in vM5LacZ-transduced hMPCs. Ceramics were seeded with hMPCs and implanted in SCID mice. Mice were sacrificed and ceramics shown were harvested 6 weeks later. Ceramics in A–G. X-Gal-stained for expression of LacZ as described in Materials and Methods, decalcified, embedded, sectioned, and counterstained as noted below. A–D (pairs A and B and C and D are each from the same cube) are from ceramics coated with vM5LacZ-transduced MPCs. E and F. Cube coated with nontransduced MPCs. MPCs and their progeny line the ceramic pores, form bone, and are trapped as osteocytes within bone, whereas the center of the pores consists of host-derived connective tissue and vasculature. A and C. Slides counterstained with Mallory Heidenhain. B and D. Higher-power views of slides counterstained with Neutral Red and show blue-stained osteocytes in bony lacunae from a cube coated with LacZ-transduced MPCs. E and F. Slides counterstained with Hematoxylin and Eosin. They do not show evidence of LacZ-expressing cells, indicating lack of endogenous β-galactosidase in human cells or from the host mouse cells that infiltrate the ceramic. Otherwise, the formation of bone is similar to the cubes analyzed from cultures of transduced MPCs. Original magnifications: B, D, and F, 200×; A, C, and E, 40×. Black arrow heads, osteocytes; OB and black arrows, osteoblast; B, bone; C, ceramic; F, fibrous tissue; Fb, fibroblast like; A, adipocytes; V, vasculature.

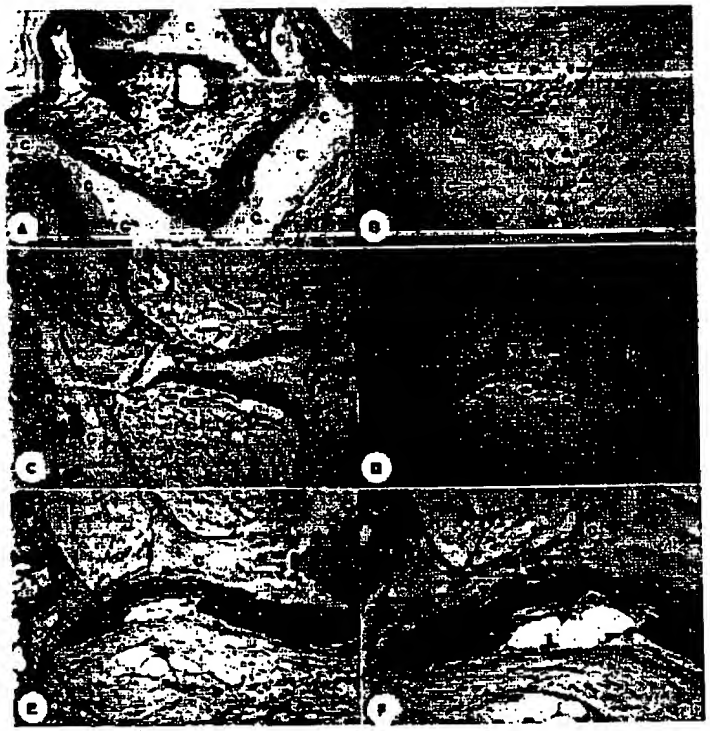

FIG. 5. See facing page for legend.

FIG. 6. Bone formation in cubes containing MPCs transduced with IL-3. Ceramic cubes were coated with MPCs transduced with vL-IL-3-SN and implantation into NOD/Lt3z/5cid/5cid mice. At 9 weeks, cubes were recovered, fixed, sectioned, and stained with Mallory Heidenhain. Bone formation is shown adherent to the ceramic of one representative cube.

TABLE 1. BONE FORMATION IN CERAMIC CUBES SEEDED WITH UNTRANSDUCED AND hIL-3-TRANSDUCED hMPCs

	Weeks after implantation								
Mouse	1	2	3	4	5	6	7	8	12
HIL-3/hMPC	0/15	0/15	0/10	9/15	7/10	6/10	4/5	7/10	4/5
hMPC	ND	0/5	0/5	4/5	ND	3/5	ND	ND	ND
No cells	0/1	0/1	ND	ND	0/1	ND	ND	ND	ND

Ratio of cubes containing bone to total cubes analyzed; five cubes per mouse.

hIL-3 expression in vL-IL-3-SN-transduced hMPCs

To determine the potential for *in vivo* production of a secreted cytokine by hMPCs implanted in ceramic cubes, hMPCs were retrovirally transduced with the human IL-3 (hIL-3) cDNA. Data shown represent three independent transductions of hMPCS followed by coating of ceramic cubes and implantation into SCID mice. *In vitro* GP + envAM12 hIL-3 producers secreted $1.9 \pm 0.01 \times 10^5$ pg of IL-3/ml per 10^6 cells per 24 hr, and hIL-3-transduced, G418-selected hMPCs secreted $0.5 \pm 0.01 \times 10^5$ pg IL-3/ml per 10^6 cells per 24 hr. vM5LacZ producers and untransduced hMPCs secreted undetectable levels of hIL-3. hIL-3-transduced hMPCs that were expanded in culture for 6 weeks, seeded into ceramic cubes, and implanted subcutaneously in NOD/LtSz-scid mice retained their osteogenic potential. MPC-IL-3 from all donors harvested from ceramic cubes beyond 4 weeks were able to produce bone *in vivo* (Table 1 and Fig. 6).

All mice implanted with IL-3-transduced cells also received an infusion of 10^7 human cord blood cells to define whether the production of hIL-3 altered maintenance of human hematopoietic cells in the mice. hIL-3 was detectable in the systemic circulation of mice up to 12 weeks after implantation. The mean level of plasma hIL-3 in mice implanted with hIL-3-MPCs was 48 ± 24 pg/ml (range 12–65 pg/ml, $n = 5$; Table 2). The mean hIL-3 level in mice implanted with untransduced hMPCs was 1.4 ± 1.9 pg/ml (values, 0, 2.7 pg/ml, $n = 2$) whereas the hIL-3 level in one mouse that was infused with human cord blood cells but was not implanted with hMPCs was 0.17 pg/ml. In both instances, this low level of IL-3 production was presumably derived from the T lymphocytes infused with the human hematopoietic cells. We were unable to evaluate whether IL-3 expression was occurring in the cells that formed bone, but, as previously noted, ceramic cubes not coated with MPCs failed to produce bone and the cell cultures of MPCs used to coat the ceramic in these experiments were selected for neo resistance and expressed IL-3 *in vitro*. Although it is possible that there was loss of IL-3 expression by some portion of these cells, it is doubtful that all cells lost the transgene, or that there was loss of osteogenic potential by IL-3 gene expression.

In other experiments (Majumdar *et al.*, submitted), we have found that MPCs do not express IL-3 mRNA or protein, indicating that the source of IL-3 is not untransduced MPCs. In mice receiving ceramic cubes with hIL-transduced MPCs, the level of IL-3 varied but did not appear to extinguish with time (Table 2). Thus, in three independent experiments, evaluated at different time points, hIL-3-transduced hMPCs retain osteogenic potential and secrete hIL-3 *in vivo*. Human hematopoietic cells (both $CD45^+$ cells and human CFUs) were found in the bone marrow at low levels (1–4% of total cells or CFUs) in these mice during the course of the 12-week experiments. However, the concentration of human $CD45^+$ cells and CFUs recovered in blood or marrow was similar in NOD/LtSz-scid mice implanted with untransduced and with IL-3-transduced MPCs. We were also unable to identify human hematopoietic cells within the ceramic cubes by immunohistologic analysis of hCD45 (data not shown). From this, we conclude that IL-3 could be released from the transduced MPCs but that these ceramic spaces did not support hematopoiesis in the NOD/LtSz-scid mice.

DISCUSSION

These data show that primary human marrow-derived mesenchymal progenitor cells capable of osteogenic differentiation *in vivo* can be retrovirally transduced, culture expanded in G418 for up to 6 weeks, and continue to express genes of interest *in vivo* while undergoing osteogenic differentiation for at least 12 additional weeks, or a total of 18 weeks after retroviral infection. These cells, termed hMPCs, because of their potential to differentiate along many mesenchymal lineages, were analyzed using the ability to form bone in the ceramic cube assay *in vivo* in SCID mice (Goshima *et al.*, 1991a,b; Dennis *et al.*, 1992, Dennis and Caplan, 1996). Although retroviral infection of marrow derived human stromal cells has been described (Nolta *et al.*, 1994), this is the first to document the ability of these cells to have osteogenic capacity *in vivo* after gene transfer. Fully differentiated osteocytes continued to express the proviral genes, indicating the permissive nature of transgene expression in these cells.

In these studies with the MPSV and LXSN retroviral vector backbones, we have shown that both the intact and spliced proviral mRNA was produced and that both the selectable marker gene, neo, and the gene of interest, either Lac Z or IL-3, could be expressed both *in vitro* and *in vivo*. The favorable transduction and expression frequency of marrow-derived hMPCs compared to many studies with hematopoietic progenitors, suggests numerous gene therapy applications. *In vivo*, transduced hMPCs continue expression of the LacZ gene 9 weeks after implantation into SCID mice. At 6 and 9 weeks, $LacZ^+$ osteoblasts and osteocytes were detected by X-Gal staining. Our laboratories have studied bone formation by MPCs from multiple species in ceramics. Variability in the amount of bone produced has been observed in these studies and it is not uncommon for a portion of the cubes coated with the same MPC preparation implanted into different SCID mice to contain no bone, presumably due to both host and donor factors. We are currently evaluating a quantitative measure for the amount of bone formed, but this assay is still being validated. Nonetheless, the amount of bone formation by transduced and non-transduced MPCs appeared the same.

Since the *in vivo* "homing" of marrow-derived stromal cells is ill-defined, we used the ceramic cube model to verify that the transduced cells would persist *in vivo* and differentiate into bone-forming cells. Most studies suggest that the stroma retains its host origin after bone marrow transplantation (Simmons *et al.*, 1987), in part because the cells appear "resistant" to preparative regimens and because so few stromal cells are actually transplanted. Keating *et al.* (1982) identified donor stromal cells (Anklesaria *et al.* 1987, 1989) have shown that a murine stromal cell line can assist reconstitution of lethally irradiated bone marrow, proving that "homing" can occur. In addition, Pereira *et al.* have shown that culture expanded murine BM-derived stromal cells have the potential for bone marrow engraftment when administered in a transplant setting to irradiated recipients (Pereira *et al.*, 1995).

Our model shows that transduction of hMPCs and subsequent implantation within an osteoconductive microenvironment is a mechanism for introducing cytokines *in vivo*. The ectopic ceramics in SCID mice are heavily vascularized so that the secreted product is not confined to the local environment but is able to reach the systemic circulation. The secretory capacity of MPCs differentiating into osteoblasts could also be utilized in gene therapy. hMPCs transduced with hIL-3 cDNA and placed within this microenvironment secreted detectable levels of hIL-3 into the systemic circulation of the NOD/LtSz-scid mouse for 12 weeks. The approximate steady-state level of hIL-3 produced per cell was 3×10^{-4} pg/ml/cell and is similar to the 2.6×10^{-4} pg/ml per cell reported after infusion of hIL-3-transduced marrow stromal cells by Nolta *et al.* (1994), although the absolute serum levels are lower because the number of transduced cells was much lower.

TABLE 2. hIL-3 LEVELS IN NOD/LtSz-scid/scid MICE IMPLANTED WITH CERAMIC CUBES SEEDED WITH hIL-3-TRANSDUCED hMPCs AS A FUNCTION OF TIME

	Weeks after ceramic cube implantation hIL-3 (pg/ml)					
Mouse	1	2	5	7	10	12
hIL-3/hMPC	65	ND	33.1	64.5	11.9	63.3
hMPC	ND	2.7	0	ND	ND	ND
HSC	ND	ND	0.17	ND	ND	ND
NOD/LtSz-scid/scid	0					

NOD/LtSz-scid/scid mice implanted ceramic cubes seeded with hIL-3-transduced hMPCs and infused with human hematopoietic cells were sacrificed at the indicated time after implantation and the hIL-3 plasma levels were quantitated by ELISA as described in Materials and Methods. The mean level of IL-3 in these mice (47 ± 24 pg/ml) was higher than in 3 mice that received untransduced MPCs with (2.7, 0 pg/ml) or without infusion of cord blood cells (0.17 pg/ml). $p < 0.05$.

These experiments were initially designed to recruit human hematopoietic cells into the spaces of the ceramic cube, thereby creating an ectopic human hematopoietic habitat. However, we were unable to identify such spaces histologically within the "humanized" ceramic cubes for CD45 staining even though human CFU were recovered from the mouse marrow. Lack of success was either due to low levels of IL-3, the fact that the mice were not irradiated to enhance hematopoietic engraftment (Krowka *et al.*, 1991; Kollmann *et al.*, 1994), or that the human hematopoietic cells could not home into the ectopic stromal and osteogenic space because connective tissue cells derived from the host filled the ceramic pores. It is also possible that the hIL-3 production did not emanate from the ceramic cube-bound MPCs. For instance, it is possible that the MPCs within the cube were not producing the hIL-3 either because the hIL-3-producing cells migrated away from the ceramic or that the hIL-3-expressing cells were unable to produce bone. However, there is no evidence that MPCs leave the ceramic, and all of the MPCs coating the ceramics were selected for proviral integration and expression, thus all contained the hIL-3 transgene. Because bone was observed in the ceramics, it would not appear that hIL-3 production precluded bone formation. It is also possible that the hIL-3 production was from the T cells infused from the cord blood cell preparation. However, in the 3 mice receiving hematopoietic cell infusions alone or with untransduced MPCs, the levels of hIL-3 ranged from 0 to 2.7 pg IL-3/ml, about 4% of the value seen in the mice receiving hIL-3-transduced MPCs. It is not surprising that the T cells would produce low levels of IL-3 in some mice but unlikely that they are the source of the high levels of IL-3 consistently observed in the mice containing ceramics with IL-3-transduced MPCs. Thus, MPCs appear to be the source of the IL-3 detected in these mice.

The ceramic cube setting creates an osteogenic environment for hMPC differentiation during which time gene expression continues. This could be used as a therapeutic strategy. Cytokines, coagulation factors, such as Factor VIII and IX, and hormones could be expressed from bone-forming cells. In some settings, cells in cubes could be implanted and later removed so that delivery of a gene product could be regulated. MPCs administered in ceramic cubes aid bone grafting and these cells could be transduced to express proteins that enhance bone healing while reducing the inflammatory response (Caplan *et al.*, 1993). A number of gene defects could be corrected in MPCs, which are then administered to specific mesenchymal sites, where they would be predicted to differentiate down defined lineages. In particular, osteogenesis imperfecta type I (OI type 1), which is characterized by brittle bones, is the manifestation of a reduction in the amount of type I collagen resulting from a functional deletion of the proα1(I) gene (Barsh *et al.*, 1985). Retroviral transduction of MPCs, like that reported for fibroblasts derived from a murine model of OI type 1 (Jaenisch *et al.*, 1983; Harbers *et al.*, 1984) could produce increased levels

of secreted type I collagen (Stacey *et al.*, 1987) and, because of their osteogenic potential, become resident in the bone. Correction of the OI defect would be more complex because mixtures of normal and abnormal collagen remain biologically abnormal although there may be potential benefit compared to the pure OI-derived collagen (Bank *et al.*, 1985).

In summary, transduction of MPCs may expand the repertoire of normal, primary cells amenable to *ex vivo* gene therapy with applications in any setting in which reconstitution of mesenchymal tissues is contemplated.

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REFERENCES

ALLAY, J., DUMENCO, L., KOÇ, O., LIU, L., and GERSON, S. (1995). Retroviral transduction and expression of the human alkyltransferase cDNA provides nitrosourea resistance to hematopoietic cells. Blood 85, 3342–3351.

ANKLESARIA, P., KASE, K., GLOWACKI, J., HOLLAND, C., SAKAKEENY, M., WRIGHT, J., FITZGERALD, T., LEE, C., and GREENBERGER, J. (1987). Engraftment of a clonal bone marrow stromal cell line *in vivo* stimulates hematopoietic recovery from total body irradiation. Proc. Natl. Acad. Sci. USA 84, 7681–7685.

ANKLESARIA, P., FITZGERALD, T., KASE, K., OHARA, A., and GREENBERGER, J. (1989). Improved hematopoiesis in anemic S1/S1^{d} mice by splenectomy and therapeutic transplantation of a hematopoietic microenvironment. Blood 74, 1446–1451.

BAB, I., PASSI-EVEN, L., GAZIT, D., SEKELES, E., ASHTON, B., PEYLAN-RAMU, N., ZIV, I., and ULMANSKY, M. (1988). Osteogenesis in *in vivo* diffusion chamber cultures of human marrow cells. Bone Min. 4, 373–386.

BARSH, G., ROUSH, C., BONADIO, J., BYERS, P., and GELINAS, R. (1985). Intron-mediated recombination may cause a deletion in an osl type I collagen chain in a lethal form of osteogenesis imperfecta. Proc. Natl. Acad. Sci. USA 82, 2870–2874.

BAUM, C., FORSTER, P., HEGEWISCH-BECKER, S., and HARBERS, K. (1994). An optimized electroporation protocol applicable to a wide range of cell lines. Biotechniques 17, 1058–1062.

BELMONT, J.W., MACGREGOR, G.R., WAGER-SMITH, K., FLETCHER, P.A., MOORE, K.A., HAWKINS, D., VILLALON, D., CHANG, S.M., and CASKEY, C.T. (1988). Expression of human adenosine deaminase in murine hematopoietic cells. Mol. Cell. Biol. 8, 5116–5125.

BOLOTIN, E., NOLTA, J., SMITH, S., DAO, M., WEINBERG, K. (1996). Gene therapy with marrow stroma transduced with IL-7 gene rapidly after murine bone marrow transplant. Blood 88, 135a.

BOESEN, D.M., MCDONAGH, K.T., BRANDT, S.J., NEY, P.A., AGRICOLA, B., BYRNE, E., and NIENHUIS, A.W. (1990). Development of a high-titer retrovirus producer cell line capable of gene transfer into rhesus monkey hematopoietic stem cells. Proc. Natl. Acad. Sci. USA 87, 3738–3742.

BRUDER, S., JAISWOL, N., and HAYNESWORTH, S. (1997). Growth kinetics, self-renewal and the osteogenic potential of purified human mesenchymal stem cells during extensive subcultivation and following cryopreservation. *J. Cell. Biochem.* 64, 272–274.

CAPLAN, A.I., FINK, D.J., GOTO, T., LINTON, A.E., YOUNG, R.G., WAKITANI, S., GOLDBERG, V.M., and HAYNESWORTH, S.E. (1993). Measurement stem cells and tissue repair. In *The Anterior Cruciate Ligament: Current and Future Concepts*. D.W. Jackson, ed. (Raven Press, Ltd., New York) pp. 405–417.

CLAPP, D., FREILE, B., SROUR, E., YODER, M., FORTNEY, K., and GERSON, S. (1995). Myeloproliferative sarcoma virus directed expression of β-galactosidase following retroviral transduction of murine hematopoietic cells. Exp. Hematol. 23, 630–638.

COURNOYER, D., SCARPA, M., MITANI, K., MOORE, K., MARKOWITZ, D., BANK, A., BELMONT, J., and CASKEY, C. (1991). Gene transfer of adenosine deaminase into primitive human hematopoietic progenitor cells. Hum. Gene Ther. 2, 203–213.

DENNIS, J., and CAPLAN, A. (1996). Analysis of the developmental potential of conditionally immortal marrow derived mesenchymal progenitor cells isolated from the H-2Kb-tsA58 transgenic mouse. Conn. Tissue Res. 35, 93–99.

DENNIS, J., HAYNESWORTH, S., YOUNG, R., and CAPLAN, A. (1992). Osteogenesis in marrow-derived mesenchymal cell porous ceramic composites transplanted subcutaneously: Effect of fibronectin and laminin on cell retention and rate of osteogenic expression. Cell Transplant. 1, 23–34.

FRIEDENSTEIN, A. (1976). Precursor cells of mechanocytes. Int. Rev. Cytol. 47, 327–339.

FRIEDENSTEIN, A. (1980). Stromal mechanisms of bone marrow: Cloning *in vitro* and retransplantation *in vivo*. In *Immunobiology of Bone Marrow Transplantation*. (Berlin, Springer-Verlag) pp. 19–29.

FRIEDENSTEIN, A., CHAILAKHJAN, R.K., and LALYKINA, K.S. (1970). The development of fibroblasts colonies in monolayer cultures of guinea pig bone marrow and spleen cells. Cell Tissue Kinet. 3, 393–403.

GERSON, S. L., RAY, K., BERGER, N.A. (1985). O^{6}-alkylguanine DNA alkyltransferase activity in human myeloid cells. J. Clin. Invest. 76, 2106–2114.

GOLDE, D., HOCKING, W., QUAN, S., SPARKES, R., and GALE, R. (1980). Origin of the human bone marrow fibroblast. Br. J. Haematol. 44, 183–187.

GOSHIMA, J., GOLDBERG, V., and CAPLAN, A. (1991a). Osteogenic potential of culture-expanded rat marrow cells as assayed *in vivo* with porous calcium phosphate ceramic. Biomaterials 12, 253–258.

GOSHIMA, J., GOLDBERG, V., and CAPLAN, A. (1991b). The osteogenic potential of culture-expanded rat marrow mesenchymal cells assayed *in vivo* in calcium phosphate ceramic blocks. Clin. Orthop. 262, 298–311.

GOSHIMA, J., GOLDBERG, V., and CAPLAN, A. (1991c). The origin of bone formed in composite grafts of porous calcium phosphate ceramic loaded with marrow cells. Clin. Orthop. 269, 274–283.

GVETNER, D.L., SHULTZ, L.D., YATES, J., APPEL, M.C., PERDRIZET, G., HESSELTON, R.M., SCHWEITZER, I., BEAMER, W.G., SHULTZ, K.L., and PELSUE, S.C. (1995). Am. J. Pathol. 146, 888–902.

HARBERS, K., KUEHN, M., DELIUS, H., and JAENISCH, R. (1984). Insertion of retrovirus into the first intron of α1(I) collagen gene leads to embryonic lethal mutation in mice. Proc. Natl. Acad. Sci. USA 81, 1504–1508.

HAYNESWORTH, S.E., and CAPLAN, A.I. (1992). Cell surface antigens on human marrow-derived mesenchymal cells are detected by monoclonal antibodies. Bone 13, 69–80.

HAYNESWORTH, S.E., GOLDBERG, V.M., and CAPLAN, A.I. (1992). Characterization of cells with osteogenic potential from human marrow. Bone 13, 81–88.

HAYNESWORTH, S.E., BABER, M., and CAPLAN, A.I. (1996). Cytokine expression by human marrow-derived mesenchymal progenitor cells *in vitro*: Effects of dexamethasone and IL-1α. J. Cell Physiol. 166, 585–592.

HOGGE, D.E., and HUMPHRIES, R.K. (1987). Gene transfer to primary human and murine hematopoietic progenitors using recombinant retroviruses. Blood 69, 611–617.

HUGHES, P., CAVES, C., HOGGE, D., and HUMPHRIES, R. (1989). High-efficiency gene transfer to human hematopoietic cells maintained in long-term marrow culture. Blood 74, 1915–1922.

HUMANSON, G. (1972). *Animal Tissue Techniques*. (San Francisco, W.H. Freeman) pp. 179–186.

JAENISCH, R., HARBERS, K., SCHNIEKE, A., LOHLER, J., CHUMAKOV, I., JAHNER, D., GROTKOPP, D., and HOFFMANN, E. (1983). Germline integration of moloney murine leukemia virus at the Mov-13 locus leads to recessive lethal mutation and early embryonic death. Cell 32, 209–216.

KEATING, A., SINGER, J., KILLEN, P., STRIKER, G., SALO, A., SANDERS, J., THOMAS, E., THORNING, D., and HALKLOW, P. (1982). Donor origin of the *in vitro* hematopoietic microenvironment after transplantation in man. Nature 298, 280–283.

KOLLMANN, T., KIM, A., ZHUANG, X., HACHAMOVITCH, M., and GOLDSTEIN H. (1994). Reconstitution of SCID mice with human lymphoid and myeloid cells after transplantation with fetal bone marrow without the requirement for exogenous human cytokines. Proc. Natl. Acad. Sci. USA 91, 8032–8036.

KROUKA, S., SARIN, S., NAMIKAWA, R., McCUNE, J., and KANESHINA, H. (1991). Human T cells in the SCID-hu mouse and phenotypically normal and functionally competent. J. Immunol 146, 3751–3756.

LAZARUS, H.L., HAYNESWORTH, S., GERSON, S.L., and CAPLAN, A.I. (1997). Human bone marrow-derived mesenchymal (stromal) progenitor cells cannot be recovered from peripheral blood progenitor cell collections. J. Hematotherapy, in press.

LAVER, J., JHANWAR, S., O'REILLY, R., and CASTRO-MALASPINA, H. (1987). Host origin of the human hematopoietic microenvironment following allogeneic bone marrow transplantation. Blood 71, 1966–1968.

LENNON, D., HAYNESWORTH, S., YOUNG, R., DENNIS, J., and CAPLAN, A. (1995). A chemically defined medium supports *in vitro* proliferation and maintains the osteochondral potential of rat marrow-derived mesenchymal stem cells. Exp. Cell Res. 219, 211–222.

LIM, B., WILLIAMS, D., and ORKIN, S. (1987). Retrovirus-mediated gene transfer of human adenosine deaminase. Expression of functional enzyme in murine hematopoietic stem cells *in vivo*. Mol. Cell. Biol. 7, 3459–3465.

MARKOWITZ, D., GOFF, S., and BANK, A. (1988). Construction and use of a safe and efficient amphotropic packaging cell line. J. Virol. 167, 400–406.

McCULLOCH, C., STRUGURESCU, M., HUGHES, P., MELCHER, A., and AUBIN, J. (1991). Osteogenic progenitor cells in rat bone marrow stromal populations exhibit self-renewal in culture. Blood 77, 1906–1911.

McIVOR, R.S., JOHNSON, M.J., MILLER, A.D., PITTS, S., WILLIAMS, S.R., VALERIO, D., MARTIN, D.W., JR., and VERMA, I.M. (1987). Human purine nucleoside phosphorylase and adenosine deaminase: Gene transfer into cultured cells and marine hematopoietic stem cells by using amoembiant amphotropic retroviruses. Mol. Cel. Biol. 7, 838–846.

MAJUMDAR, M.P., HAYNESWORTH, S.E., THIEDE, M.A., MARSHAK, D.R., CAPLAN, A.L., and GERSON, S.L. (1995). Colonie expanded human mesenchymal stem cells (MSCs) express cytokines and support hematopoiesis in vitro. Blood 86, 494a.

METCALF, D., and NICOLA, N. (1983). Tissue localization and fate in mice of injected multipotential colony-stimulated factor. Proc. Natl. Acad. Sci. USA 80, 3160–3164.

NAKAHARA, H., GOLDBERG, V., and CAPLAN, A. (1991). Culture-expanded human periosteal-derived cells exhibit osteochondral potential *in vivo*. J. Orthop. Res. 9, 465–478.

NAKAHARA, H., GOLDBERG, V., and CAPLAN, A. (1992). Culture-expanded periosteal-derived cells exhibit osteochondrogenic potential in porous calcium phosphate ceramics *in vivo*. Clin. Orthop. 276, 291–298.

NOLTA, J., HANLEY, M., and KOHN, D. (1994). Sustained human hematopoiesis in immunodeficient mice by cotransplantation of marrow stroma expressing human interleukin-3: Analysis of gene transduction of long-lived progenitors. Blood 83, 3041–3051.

OWEN, M. (1988). Marrow stromal stem cells. J. Cell Sci. 10, 63–76.

OWEN, M., CAVE, J., and JOYNER, C. (1987). Clonal analysis *in vitro* of osteogenic differentiation of marrow CFU-F. J. Cell Sci. 87, 731–738.

PEREIRA, R., HALFORD, K., O'HARA, M., LEEPER, D., SOKOLOV, B., POLLARD, M., BAGASRA, O., and PROCKOP, D. (1995). Cultured adherent cells from marrow can serve as long-lasting precursor cells for bone, cartilage, and lung in irradiated mice. Proc. Nat. Acad. Sci. USA 92, 4857–4861.

PROCKOP, D.J. (1997). Marrow stromal cells as stem cells for nonhematopoietic tissues. Science 276, 71–74.

SAMBROOK, J., FRITSCH, E., and MANIATIS, T. (1989). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY).

SANES, J., RUBENSTEIN, J., and NICOLAS, J-F. (1986). Use of a recombinant retrovirus to study post-implantation cell lineage in mouse embryos. EMBO J. 5, 3133–3142.

SIMMONS, P.J., PRZEPIORKA, D., THOMAS, E.D., and TOROK-STORB, B. (1987). Host origin of marrow stromal cells following allogeneic marrow transplantation. *Nature* 328, 429–432.

STACEY, A., MULLIGAN, R., and JAENISCH, R. (1987). Rescue of type I collagen-deficient phenotype by retroviral-vector-mediated transfer of human procol(I) collagen gene into Mov-13 cells. *J. virol.* 61, 2549–2554.

WAKITANI, S., SAITO, T., and CAPLAN, A.L. (1995). Myogenic cells derived from rat bone marrow mesenchymal stem cells exposed to 5-azacytidine. Muscle Nerve 18, 1417–1426.

WILLIAMS, D.A. (1990). Expression of introduced genetic sequences in hematopoietic cells following retroviral-mediated gene transfer. Hum. Gene. Ther. 1, 229–239.

WILLIAMS, D., ORKIN, S., and MULLIGAN, R. (1986). Retrovirus-mediated transfer of human adenosine deaminase gene sequences into cells in culture and into murine hematopoietic cells *in vivo*. Proc. Natl. Acad. Sci. USA 83, 2566–2570.

WILSON, J., DANOS, O., GROSSMAN, M., RAULET, D., and MULLIGAN, R. (1990). Expression of human adenosine deaminase in mice reconstituted with retrovirus-transduced hematopoietic stem cells. Proc. Natl. Acad. Sci. USA 87, 439–443.

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